Folate supplementation reduces serum Hsp70 levels in patients with type 2 diabetes

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Abstract Type 2 diabetes patients are subject to oxidative stress as a result of hyperglycemia. The aim of this study was to determine whether administration of the antioxidant folic acid, previously shown to reduce homocysteine levels, would reduce circulating levels of Hsp70 while improving the condition of type 2 diabetes patients with microalbuminuria. Plasma homocysteine fell from pretreatment values of 12.9 to 10.3 μM ($P < 0.0001$). The urine albumin-creatinine ratio fell from 12.4 to 10.4 mg/mM ($P = 0.38$). Pretreatment Hsp70 levels were higher in patients not taking insulin (5.32 ng/mL) compared with those on insulin (2.44 ng/mL) ($P = 0.012$). Folic acid supplementation resulted in a significant fall in Hsp70 (5.32 to 2.05 ng/mL) ($P = 0.004$). There was no change in Hsp70 in those receiving insulin. Folic acid supplementation in non-insulin-treated type 2 diabetes patients, therefore, resulted in a fall in Hsp70, reflecting an improvement in oxidative stress. The data shows that improvement in homocysteine status can lead to a reduction in Hsp70, indicating the possibility of its use as a marker for severity of disease.

INTRODUCTION

Type 2 diabetes is commonly associated with cardiovascular complications, which are responsible for the deaths of up to 80% of patients (Spanheimer 2001). The cause of these vascular complications in patients with type 2 diabetes may be the oxidative stress resulting from hyperglycemia (Spanheimer 2001; Sampson et al 2002). Another potential source of oxidative stress is the elevated homocysteine levels often found in type 2 diabetes (Stehouwer et al 1999). High levels of homocysteine are associated with endothelial cell dysfunction and are a significant risk factor for vascular disease (Boushey et al 1995; Hofmann et al 2001; Wald et al 2002). Plasma total homocysteine concentration is a significant predictor of mortality in type 2 diabetes patients with or without microalbuminuria (Stehouwer et al 1999). Despite the association of homocysteine levels with endothelial function and vascular disease, it is not clear whether homocysteine

increases are causative or are an effect of oxidative stress (Doshi et al 2002; Moat et al 2003).

Folic acid supplementation has been shown to decrease homocysteine levels (Boushey et al 1995; Brattstrom 1996; Homocysteine Lowering Trialists' Collaboration 1998) and increase plasma total glutathione levels (Arnadottir et al 2000). In asymptomatic healthy subjects with hyperhomocysteinemia, folate treatment decreases homocysteine levels and leads to significantly improved endothelial cell function (Woo et al 1999). Folic acid supplementation, therefore, has potential as a treatment to reduce vascular disease in diabetes patients.

Vascular damage is usually indicated by an increase in the urinary albumin excretion rate (AER) (Hofmann et al 1998; Lanfredini et al 1998; Harvey et al 1999), although the urinary albumin-creatinine ratio (ACR) is a preferable measure (Harvey et al 1999). Plasma homocysteine shows a positive correlation with AER in type 1 (Hofmann et al 1998) and type 2 (Lanfredini et al 1998) diabetes patients, suggesting that a reduction in homocysteine would possibly reduce AER and, therefore, improve the condition of patients with microalbuminuria.

Hsp70 expression increases as a result of oxidative

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stress in addition to heat shock and other environmental stresses (Lindquist and Craig 1988; Latchman 1999). Hsp70 protein levels (Marini et al 1996) and *hsp70* messenger ribonucleic acid (mRNA) expression (Hunter-Lavin 2003) are increased in peripheral blood mononuclear cells (PBMCs) on exposure to hydrogen peroxide. Hsp70 has also been found in the serum of normal individuals (Pockley et al 1998) and those with vascular disease (Wright et al 2000). The increase in *hsp70* mRNA in response to oxidative stress is greater in Chinese hamster ovary cells depleted of glutathione compared with glutathione-replete cells (Sierra-Rivera et al 1994). Glutathione is a product of homocysteine metabolism and an important antioxidant. Therefore, in a cellular system Hsp70 levels are correlated to oxidative stress. Type 2 diabetes patients are subject to oxidative stress (Spanheimer 2001; Sampson et al 2002), and it is therefore not surprising to find elevated Hsp70 concentrations in the PBMCs of these patients (Yabunaka et al 1995).

This study investigates the effects of folic acid supplementation on type 2 diabetes patients with microalbuminuria. Previous studies of the effects of oxidative stress on Hsp70 have concentrated on increases in the protein in response to increased stress and are mainly in vitro experiments. Previous studies have shown that folate treatment reduces serum homocysteine, thereby reducing oxidative stress in vivo. We hypothesize that this will result in a reduction of Hsp70 levels in serum.

MATERIALS AND METHODS

Patients

Local Ethics Committee permission was obtained for this study. Informed written consent was obtained from each patient involved.

Type 2 diabetes patients with microalbuminuria were recruited from the diabetes register. Twenty-six patients were recruited in total with an age range of 44–68 years, a mean of 59 years, and was composed of 25 men and 1 women. Sex bias was unintentional and due to ease of recruitment. Twelve of the patients were on insulin.

Patients were given 10 mg folic acid daily, for a 3-month period. Fasting blood samples and early morning urine samples were taken before and after treatment. Blood samples were separated and frozen at -20°C within 1 hour of collection. Urine albumin was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) using rabbit antihuman serum albumin immunoglobulin G (IgG) and horseradish peroxidase-conjugated rabbit antihuman serum albumin IgG (Dako Ltd, Ely, UK) (Harvey et al 1999). Creatinine was measured by an automated analyzer (Beckman LX20, Beckman-Coulter, UK Ltd, High Wycombe, UK).

Red cell folate was measured on an automated chemiluminescence analyser (ACS:180 SE, Bayer plc, Newbury, UK). Amino-thiol samples were collected into ethylenediaminetetraacetic acid, separated, and frozen at -20°C within 1 hour of collection. Homocysteine and glutathione were assayed using a modified protocol (Ranganath et al 2001) for separation of homocysteine and glutathione from other amino-thiols on an automated HPLC system (DS30 Analyser, Drew Scientific Group plc, Barrow in Furness, UK).

Serum Hsp70, antihuman Hsp70, and antihuman Hsp60

Hsp70 was quantified in undiluted serum using an Hsp70 ELISA Kit (EKS-700, StressGen Biotechnologies, Victoria, British Columbia, Canada) in accordance with the manufacturers' instructions. Antihuman Hsp70 (EKS-750, StressGen Biotechnologies) and antihuman Hsp60 (EKS-650, Stressgen Biotechnologies) were measured in serum samples (1/1000) in accordance with the manufacturers' instructions.

Final absorbances were measured at 450 nm (MRX II Microplate Photometer, Dynex Technologies, Thermo Life Sciences, Basingstoke, UK).

Statistical methods

Nonparametric statistical testing was used. Data are represented as medians with interquartile ranges. The Wilcoxon matched pairs test was used for paired comparisons and the Mann-Whitney *U*-test for unpaired comparisons. Correlations were assessed using the Spearman correlation coefficient (Statistica 6.0, Statsoft Ltd., Bedford, UK).

RESULTS

Folate treatment resulted in a significant increase in red cell folate from 331 to 793 $\mu\text{g/L}$ ($P < 0.0001$), a decrease in plasma homocysteine from 12.9 to 10.3 μM ($P = 0.0001$), and an increase in plasma glutathione from 1.3 to 1.7 μM ($P = 0.016$) (Table 1). There was a decrease in urine ACR from 12.9 to 10.4 mg/mM, although this did not reach significance (Table 1). However, the reduction in microalbuminuria, measured as ACR, did correlate with increased red cell folate ($P = 0.001$).

Folic acid treatment resulted in an overall decrease in Hsp70 from 4.32 to 2.06 ng/mL ($P = 0.01$) (Fig 1). In pretreatment samples, Hsp70 was higher in patients treated with oral hypoglycemic agents (5.32 ng/mL) compared with those on insulin (2.44 ng/mL) ($P = 0.012$) (Fig 2). Folic acid supplementation resulted in a significant fall in Hsp70 in those not taking insulin from 5.32 to 2.05 ng/mL ($P = 0.004$) (Fig 2). There was no signifi-

Table 1 RBC folate, homocysteine, glutathione, and urine ACR before and after folic acid treatment

	All patients (n = 26)	Noninsulin (n = 14)	Insulin (n = 12)	P value insulin vs noninsulin
RBC folate ($\mu\text{g/L}$)				
Baseline	331 (256–390)	279 (227–341)	377 (331–421)	0.017
After treatment	793 (631–919)	737 (623–881)	897 (793–943)	
P value	<0.0001	0.003	0.04	
Plasma homocysteine (μM)				
Baseline	12.9 (10.5–14.7)	11.8 (9.7–14.8)	13.9 (11.4–14.6)	
After treatment	10.3 (8.7–12.5)	9.9 (7.0–11.6)	12.1 (9.0–14.1)	
P value	0.0001	0.001		
Plasma glutathione (μM)				
Baseline	1.3 (1–1.7)	1.2 (1.0–1.7)	1.6 (1.1–1.8)	
After treatment	1.7 (1.3–1.9)	1.8 (1.3–1.9)	1.7 (1.4–2.7)	
P value	0.016			
HbA _{1c} (%)				
Baseline	8.4 (7.6–9.6)	7.8 (6.7–9.9)	8.4 (8.2–9.4)	
After treatment	9.2 (7.9–9.9)	9.4 (6.8–10.3)	9.0 (8.6–9.6)	
P value				
Urine albumin/creatinine ratio (mg/mM)				
Baseline	12.9 (4.1–17.9)	7.5 (3.3–16.4)	16.6 (8.0–18.3)	
After treatment	10.4 (4.8–16.0)	8.6 (4.8–14.1)	12.9 (4.7–16.7)	
P value				

Results are shown as medians (interquartile ranges) and *P* value (Wilcoxon signed rank test). Only those *P* values that reached significance are shown. RBC, red blood cell; ACR, albumin-creatinine ratio; and HbA_{1c}, glycosylated hemoglobin.

Fig 1. The effect of folic acid treatment on serum Hsp70 levels. Type 2 diabetes patients were given 10 mg/day folic acid for 3 months. Results are shown as median, boxes—interquartile ranges, error bars—full range, *n* = 26.

cant change in Hsp70 after folic acid treatment in insulin-treated patients (Fig 2). Red cell folate was higher in the insulin group (377 $\mu\text{g/L}$) compared with the noninsulin group (279 $\mu\text{g/L}$) before treatment (*P* = 0.017) (Table 1). Pretreatment glycosylated hemoglobin (HbA_{1c}) did not differ between non-insulin-treated and insulin-treated patients (7.8% compared with 8.4%) (Table 1). HbA_{1c} was

not significantly affected by folate treatment in either group (Table 1).

Folic acid supplementation did not give rise to any significant difference in antibodies to Hsp70 or Hsp60 (Fig 3). Antibodies to Hsp70 were constantly at higher levels than those to Hsp60 (Fig 3). Patients taking metformin had significantly lower pretreatment levels of anti-Hsp60 than those in patients receiving other treatments (*P* = 0.025) (Fig 4).

DISCUSSION

An increasing number of studies are showing that Hsp60 and Hsp70 are present in serum and are elevated in a number of disease conditions (Pockley et al 1998; Pockley 2003). This article aims to assess whether serum Hsp70 can be used as a marker of vascular health.

Folate supplementation in this study resulted in a decrease in plasma homocysteine, and an increase in glutathione. These changes suggest that there has been a decrease in the oxidative stress, which may explain the observed decrease in serum Hsp70 in the non-insulin treated group. Three patients showed increased homocysteine levels after folate treatment, suggested to be due to an insufficiency of vitamin B6 (Child et al 2004). These patients were also the only individuals who exhibited an increase in Hsp70 levels, suggesting the possibility of a specific link between homocysteine and Hsp70 in addition to the link between Hsp70 and oxidative stress in general.

Fig 2. Comparison of Hsp70 levels in insulin ($n = 12$) and non-insulin ($n = 14$)-treated patients before and after folic acid treatment. Type 2 diabetes patients were given 10 mg/day folic acid for 3 months. Results are shown as median, boxes—interquartile ranges, error bars—full range.

Fig 3. Comparison of antibody levels with Hsp70 and Hsp60 before and after folic acid treatment. Type 2 diabetes patients were given 10 mg/day folic acid for 3 months. Results are shown as median, boxes—interquartile ranges, error bars—full range, $n = 26$.

Serum antibody levels to Hsp70 and Hsp60 were unchanged by folate treatment, although these are measurements of total IgA, IgG, and IgM and it is possible that reciprocal shifts may have occurred. Anti-Hsp60 levels in this study were higher in patients not taking metformin, which may indicate an autoimmune component to their condition or be linked to the action of metformin in reducing the risk of cardiovascular disease (Grant 2003).

There has been some discussion on whether homocysteine levels can indicate the degree of oxidative stress that PBMCs and endothelial cells have experienced. It has been argued that in vivo studies showing a decrease in homocysteine and decrease in cellular damage are not due to changes in oxidative stress (Doshi et al 2002; Moat et al 2003). The data on folate supplementation could be interpreted as a direct effect of folate on cells rather than an indirect effect through homocysteine (Doshi et al 2002;

Fig 4. Comparison of anti-Hsp60 levels between Type 2 diabetes patients on metformin and those not on metformin. Results are shown as median, boxes—interquartile ranges, error bars—full range, $n = 26$.

Moat et al 2003). It is interesting that before the trial red blood cell folate was higher and Hsp70 was lower in the insulin group, compared with the noninsulin group, suggesting that there may be a direct link between folate and Hsp70.

Oxidative stress causes an increase in Hsp70 in lymphocytes (Marini et al 1996). Hsp70 may have a protective role against oxidative stress as elevated Hsp70 levels induced by heat shock decrease islet cell sensitivity to nitric oxide toxicity (Bellmann et al 1997; Rothe and Kolb 1999; Burkart et al 2000). In contrast to studies in blood, intramuscular *Hsp72* mRNA is lower in patients with type 2 diabetes as is Hsp32—also known as heme oxygenase-1 (HO-1) (Bruce et al 2003). HO-1 is induced by oxidative stress in cells (Marini et al 1996; Bruce et al 2003). Homocysteine, itself, causes a decrease in Hsp70 production by cultured neuronal cells (Althausen and Paschen 2000). The increase in Hsp70 expression resulting from oxidative stress is blocked by homocysteine in endothelial cells (Outinen et al 1998). The relationship between homocysteine, oxidative stress, and Hsp expression is clearly not

simple, and both Hsp70 and Hsp32 would be worth considering in any future studies.

Despite the fact that folate supplementation has been shown to reduce plasma homocysteine, we were not able to see the significant reduction in endothelial function (measured here as ACR) found in other studies (Woo et al 2002). Folate supplementation as low as 0.5 mg/day has resulted in a reduction of plasma homocysteine, although the dosage range used in studies has varied considerably (0.5–10 mg/day) (Homocysteine Lowering Trialists' Collaboration 1998). Here, we have used 10 mg/day, reflecting the concentration required to have an effect on endothelial function (Woo et al 1999). The lack of significant effect may be the relatively short length of the study, and long-term folate supplementation may result in a significant improvement in ACR. The extent of endothelial improvement is lessened in patients with predialysis renal failure (Thambyrajah et al 2000), suggesting that the timing of folate supplementation relative to disease progression could be critical.

The source of serum Hsp70 has not been identified and is potentially a combination of vascular cells and PBMCs. The release of Hsp70 from these cells may be due to damage or through a secretory pathway (Hightower and Guidon 1989; Liao et al 2000). The known interaction of Hsps with the cells of the immune system (Pockley 2003) make this an area of particular importance.

Folate supplementation did not result in a significant decrease in ACR, whereas in patients not treated with insulin therapy, Hsp70 levels were reduced. This suggests that Hsp70 may be a more sensitive measure of vascular health than ACR. Hsp70 may therefore be a suitable marker of the severity of the clinical condition and may be useful in the monitoring of type 2 diabetes as well as other diseases associated with oxidative stress, such as cardiovascular disease. It is clear that further work is needed to determine the source of Hsp70 in serum, whether folate is directly or indirectly causing the change in Hsp70, and the role of homocysteine in vascular disease.

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